PATENT
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In re Application of: **Heinrich Haas *et al.***	)	Confirmation No: **3937**
Application No.: **10/525,384**	)	Group Art Unit: **1618**
Filed: **February 23, 2005**	)	Examiner: **P.W. Dickinson**
For: **Non-Vesicular Cationic Lipid Formulations**	)	Date: **September 23, 2009**

RESPONSE TO NOTICE OF NON-COMPLIANT AMENDMENT

This paper responds to the Notice of Non-Compliant Amendment, mailed August 25, 2009, time for response to which expires September 25, 2009.

The Notice alleges that the elected compound of claim 53, N-[1-(2,3-diacyloxy)propyl]-N-N-N-trimethylammonium, is not an actual compound because "2,3-diacyloxy" cannot lie on the 1 position of the propyl group. Applicants respectfully point out that the recitation of "2,3-diacyloxy" indicates that the two acyloxy groups of the elected compound, N-[1-(2,3-diacyloxy)propyl]-N-N-N-trimethylammonium, are at positions 2 and 3 of the propyl group and that the nitrogen of the ammonium group is at the 1 position of the propyl group. Moreover, the attached reference of Felgner (Felgner *et al.*, PNAS, 1987, 84: 7413-7417) provides evidence that N-[1-(2,3-diacyloxy)propyl]-N-N-N-trimethylammonium is a name for an actual compound (see abstract and fig. 1). Felgner shows that N-[1-(2,3-dioleyloxy)propyl]-N-N-N-trimethylammonium, which is DOTMA, has the structure shown in figure 1. The "2,3-dioleyloxy" of DOTMA replaces the "2,3,-diacyloxy" of N-[1-(2,3-diacyloxy)propyl]-N-N-N-trimethylammonium, since oleyloxy is a acyloxy. As shown in figure 1 of Felgner, the two oleyloxy groups are at positions 2 and 3 of the propyl group and that the nitrogen of the ammonium group is at the 1 position of the propyl group. Accordingly, the elected compound of claim 53 correspond to an actual compound.

If there are any additional fees due in connection with the filing of this response, please charge the fees to our Deposit Account No. 50-0310. If a fee is required for an extension of time

under 37 C.F.R. §1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,
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Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure

(liposomes/cationic lipid vesicles/gene transfer)

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ABSTRACT **A DNA-transfection protocol has been developed that makes use of a synthetic cationic lipid, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA). Small unilamellar liposomes containing DOTMA interact spontaneously with DNA to form lipid–DNA complexes with 100% entrapment of the DNA. DOTMA facilitates fusion of the complex with the plasma membrane of tissue culture cells, resulting in both uptake and expression of the DNA. The technique is simple, highly reproducible, and effective for both transient and stable expression of transfected DNA. Depending upon the cell line, lipofection is from 5- to >100-fold more effective than either the calcium phosphate or the DEAE-dextran transfection technique.**

Under appropriate conditions, eukaryotic cells can take up exogenous DNA, and a portion of this DNA becomes localized in the nucleus. The phenomenon has been exploited to obtain both transient and stable expression of various genes (1–11). However, due in part to the size and charge of DNA and to the multitude of enzymatic and membrane barriers imposed by the cell, the spontaneous entry of intact DNA into the cell and its subsequent expression in the nucleus is a very inefficient process. For this reason, a wide variety of methods have been developed to facilitate this process. These methods include the use of polycations (1–3), calcium phosphate (4, 5), liposome fusion (6), retroviruses (7), microinjection (8), electroporation (9), and protoplast fusion (10). However, all of these methods suffer from one or more problems related to either cellular toxicity, poor reproducibility, inconvenience, or inefficiency of DNA delivery.

We have synthesized a cationic lipid that forms liposomes. We show here that these liposomes interact with DNA spontaneously, fuse with tissue culture cells, and facilitate the delivery of functional DNA into the cell. The technique is simple, highly reproducible, and more efficient than some other, commonly used procedures.

METHODS

Cells and Media. The cell line COS-7 (ATCC CRL 1651) is a derivative of the simian kidney cell line CV1 (ATCC CCL 70) transformed with a mutant of simian virus 40 (SV40) (12). ψ-2 is a murine fibroblast line that stably expresses a packaging-deficient retrovirus; it is used, most often, for the production of retroviral vector stocks (13). MSN610.2 is a glucocorticoid receptor-deficient subclone of the mouse mammary tumor virus (MMTV)-infected, rat HTC hepatoma-derived cell line MSC.1 (14). JZ.1 is an HTC hepatoma cell line containing one integrated copy of MMTV (15). L-M(TK$^-$) cells (ATCC CCL 1.3) are derived from murine L cells and are thymidine kinase-deficient (16). The TA1 cell line is derived from the murine fibroblast cell line 10T½ (17). All cells were grown on plastic tissue culture plates in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a 10% CO_2, 37°C incubator, except TA1 cells, which were grown in basal minimal essential medium with 10% fetal bovine serum in a 5% CO_2, 37°C incubator.

DOTMA Synthesis and Liposome Preparation. DOTMA {*N*-[1-(2,3,-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride} was prepared as outlined in Fig. 1. A mixture of 3-(dimethylamino)-1,2-propanediol (Aldrich; 1.19 g, 10 mmol), potassium *tert*-butoxide (3.36 g, 30 mmol), and oleyl *p*-toluenesulfonate (12.7 g, 30 mmol) in xylenes (50 ml) was stirred at room temperature and reduced pressure (30 torr; 1 torr = 133 Pa) for 30 min and then was heated to 50°C with stirring for an additional 15 min. The reaction vessel was purged with nitrogen, and the mixture was heated to reflux (≈140°C) for 3 hr. After cooling, the reaction mixture was diluted with hexane (100 ml), and the resulting solution was extracted with water (twice, 50 ml each). The organic layer was concentrated, and the residue was chromatographed over silica gel by elution with a mixture of hexanes and diethyl ether (1:2) to afford the intermediate 2,3-dioleyloxy-1-(dimethylamino)propane as a colorless oil. Quaternization was carried out by condensation of methyl chloride (50 ml) into a Parr pressure apparatus cooled to −78°C and containing this compound (10 g). The sealed vessel was heated behind a safety shield at 70°C for 48 hr. After cooling to 0°C, the reaction vessel was opened, and the excess methyl chloride was allowed to evaporate under a stream of nitrogen in a well-ventilated hood. The crude residue was recrystallized from acetonitrile to afford DOTMA as an off-white solid, mp 35–38°C. Full details of the synthesis of DOTMA and analogs will be published elsewhere (T.R.G. and P.L.F.).

A solution of dioleoyl phosphatidylethanolamine (PtdEtn; 10 mg) and DOTMA (10 mg) in chloroform (1 ml) was evaporated to dryness under a stream of nitrogen, and residual solvent was removed under vacuum overnight. Liposomes were prepared by resuspending the lipids in deionized water (2 ml) and sonicating to clarity in a closed vial. Sterile preparations of liposomes are stable for at least 6 months at 4°C.

DOTMA-Mediated Transfection of Cells (Lipofection). Plasmid DNAs were purified by the method of Birnboim and Doly (18), with subsequent removal of high molecular weight RNA by precipitation with 2.5 M LiCl and banding in CsCl

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Abbreviations: DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; CAT, chloramphenicol acetyltransferase; PtdEtn, dioleoyl phosphatidylethanolamine; PtdCho, dioleoyl phosphatidylcholine; HBS, Hepes-buffered saline; SV40, simian virus 40.

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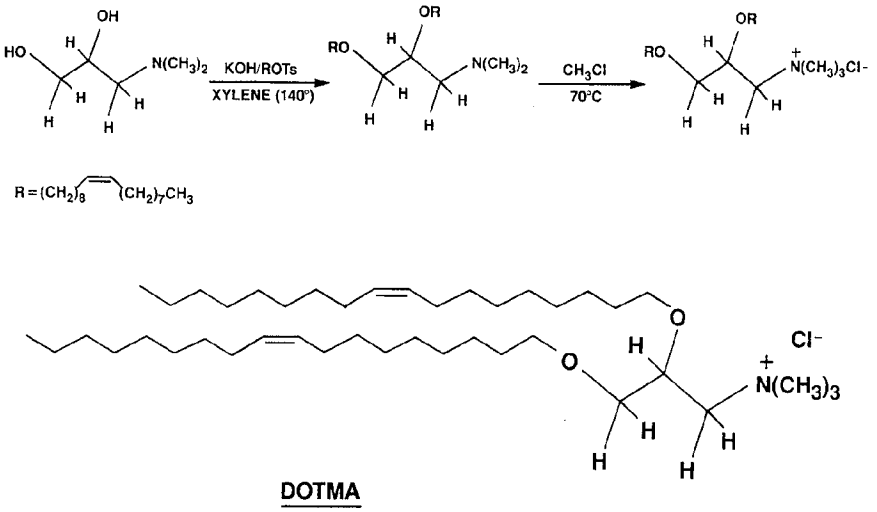

DOTMA

FIG. 1. Chemical synthesis of DOTMA. ROTs, oleyl *p*-toluenesulfonate.

gradients. Plasmids pSV2cat (19) and pSV2neo (20) have been described. pZipneoSVX is a retroviral vector that encodes a neomycin-resistance gene (7). pZipC5a and pMSGc5a were derived from pZipneoSVX and pMSG (Pharmacia), respectively, by the insertion, in the antisense orientation, of a cDNA isolated from adipogenic cells (clone 5 in ref. 17).

The details of individual transfections are given in *Results*. A general protocol for transfections is given below.

Formation of the lipid–DNA complex. DNA and lipid were each diluted to 1.5 ml with Hepes-buffered saline (HBS: 150 mM NaCl/20 mM Hepes, pH 7.4) and then mixed. The lipid–DNA complexes formed immediately. A typical transfection used 1–10 μg of DNA and 100 μg of total lipid (PtdEtn/DOTMA, 1:1 by weight).

Treatment of cells. Just-confluent cells in 100-mm tissue culture plates were washed twice with 5 ml of HBS and 3 ml of the lipid–DNA mixture was added. The cells were incubated for 3–5 hr at 37°C and then 10 ml of Dulbecco's modified Eagle's medium with 10% fetal bovine serum was added. After incubation for 16 hr at 37°C, the medium was replaced with 10 ml of fresh medium, and the cells were harvested by scraping 2–3 days later. Cell extracts were prepared and chloramphenicol acetyltransferase (CAT) assays were performed as described (21). For stable transfections, 50%-confluent cells were treated as above, except that 2 days after transfection, cells were passaged and grown in selective medium for either neomycin resistance (0.4 mg of G418 per ml) or expression of *Escherichia coli* xanthine (guanine) phosphoribosyltransferase (22, 23).

Transfection by Calcium Phosphate Precipitation and by DEAE-Dextran. Cells were transfected with calcium phosphate-precipitated DNA as described (4), with the addition of a glycerol shock (24). Cells were transfected by the DEAE-dextran method as described (21).

Staining of Cells with Rhodamine-Conjugated Lipid. A solution of DOTMA (10 mg), PtdEtn-rhodamine (Avanti Polar Lipids; 0.2 mg), and either PtdEtn (10 mg) or dioleoyl phosphatidylcholine (PtdCho; 10 mg) in chloroform (1 ml) was evaporated to dryness under a stream of nitrogen, and residual solvent was removed under vacuum overnight. Liposomes were prepared by resuspending the lipids in deionized water and sonicating to clarity in a closed vial. Fluorescent lipid–DNA complexes were prepared from these liposomes by mixing 0.5 ml of liposomes (0.1 mg of total lipid per ml in HBS) and 0.5 ml of pSV2cat DNA (0.02 mg/ml in HBS). The complexes (5 μg) were added to mouse L cells (10^5) that had been seeded onto microscope slides containing 2 × 2-cm wells. After a 4-hr incubation the cells were washed with HBS and examined by epifluorescence microscopy.

RESULTS

Formation of Lipid–DNA Complexes. Cationic lipid vesicles might be expected to have the desirable properties both of cationic mediators of DNA transfection (e.g., spontaneous complex formation with DNA and the cell surface) and of liposome-mediated transfection (rapid fusion and uptake of the DNA). However, there are no widely available cationic, bilayer-forming lipids that give rise to physically stable liposomes. Therefore, we designed and synthesized the cationic lipid DOTMA (Fig. 1), which, either alone or in combination with neutral phospholipids, spontaneously forms multilamellar liposomes that can be sonicated to form small unilamellar vesicles (data not shown). The rectangular array of the parallel alkyl chains may be a significant factor contributing to the formation and stability of DOTMA bilayers, as has been shown for other lipids (25). The characterization of these liposomes will be presented in detail elsewhere.

DNA interacts spontaneously with solutions of DOTMA to form lipid–DNA complexes. This complex formation presumably is due to ionic interactions between the positively charged group on the DOTMA molecule and the negatively charged phosphate groups on the DNA. Complex formation was examined by sucrose density gradient centrifugation (data not shown). In the absence of lipid, DNA migrated to the bottom of the gradient, whereas lipid, in the absence of DNA, floated at the top. When lipid was mixed with DNA at a ratio of 5:1 (wt/wt), all the DNA migrated with the lipid. The association of 100% of the DNA with DOTMA after gentle mixing contrasts with conventional liposome encapsulation procedures, which usually entrap less than 10% of the DNA, require an additional purification step to remove unencapsulated DNA, or involve potentially destructive conditions such as vigorous agitation or sonication (6).

Fusion of Lipid–DNA Complexes with Cells. We speculated that the positively charged lipid DOTMA would not only interact with DNA to form a complex but would also cause the complex to bind to tissue culture cells and possibly fuse with the plasma membrane. Incorporation of rhodamine-conjugated PtdEtn into the DNA–lipid complex allows one to follow the fate of the complex as it interacts with tissue culture cells (Fig. 2 *Left*). Fluorescence microscopy revealed

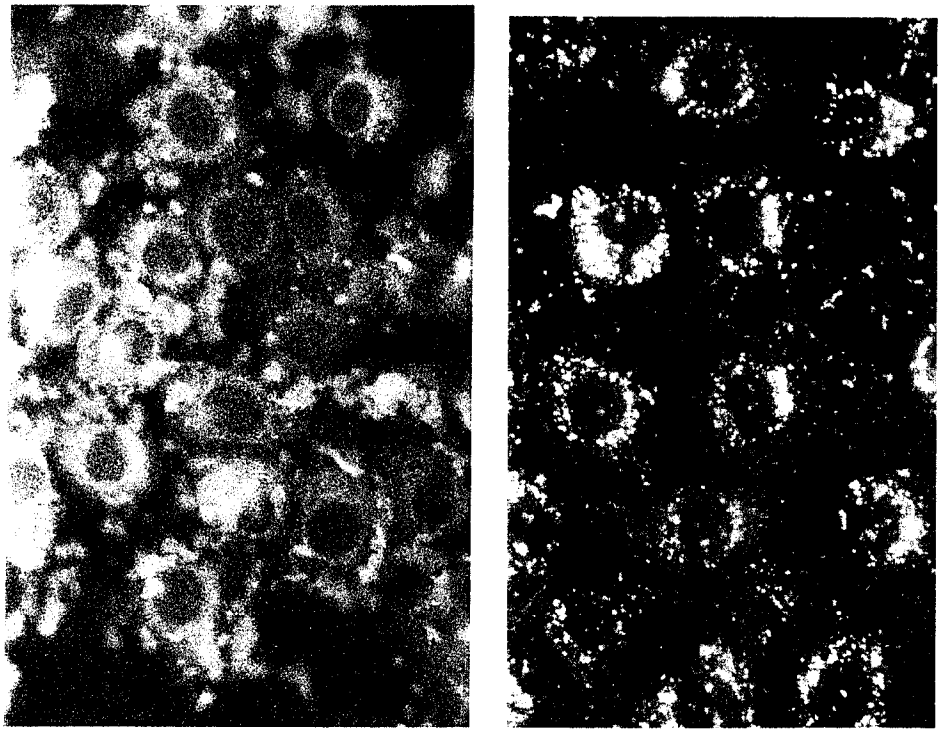

FIG. 2. Mouse L cells were seeded onto microscope slides containing 2 × 2-cm wells. The following day the cells were washed with transfection buffer (HBS), exposed to 5 μg of lipid–DNA complexes (containing 1% of the total lipid as rhodamine-conjugated PtdEtn) in 1 ml of transfection buffer, and incubated at 37°C. After various times, the cells were washed and examined by epifluorescence microscopy. The micrographs represent cells incubated for 4 hr with PtdEtn/DOTMA, 1:1 (*Left*) or PtdCho/DOTMA, 1:1 (*Right*).

that the complexes fuse with the cell membrane and that the fluorescent lipid diffuses throughout the intracellular membranes. Further, the intensity of cell-associated fluorescence increases with time, reaching a maximum after 4 hr, when virtually all the cells are fluorescently labeled. Our standard transfection mixture contains DOTMA and a neutral lipid, PtdEtn, in a 1:1 (wt/wt) ratio (see *Methods*). The fusogenic capabilities of the complex can be controlled to a certain extent by the choice of the neutral lipid used to form the complex. For instance, when PtdCho is substituted for PtdEtn, fusion of the complex with the cell membrane is inhibited and a punctate fluorescence is seen on tissue culture cells (Fig. 2 *Right*). These results are what one might predict from the known fusogenic properties of PtdEtn (26).

Optimization of the Transfection Protocol. Cells transfected with the plasmid pSV2cat express CAT enzyme activity that can be measured in cell extracts 2 days after transfection. The lipid transfection technique was optimized for this kind of transient transfection assay, using two monkey kidney cell lines, CV-1 and COS-7. COS-7 cells are often used for transient transfection assays, as they produce SV40 large tumor (T) antigen, which allows replication of plasmids, such as pSV2cat, containing an SV40 origin of replication (12). CV-1 is the parental cell line, which does not produce T antigen and in which pSV2cat cannot replicate.

Concentration of DNA. Only 1 μg of pSV2cat is required for optimal transfection of COS-7 cells. Furthermore, transfection efficiency is relatively insensitive to a broad range of DNA concentrations (Fig. 3*A*); only a 2-fold difference in CAT activity is observed when 0.2–40 μg of pSV2cat is used.

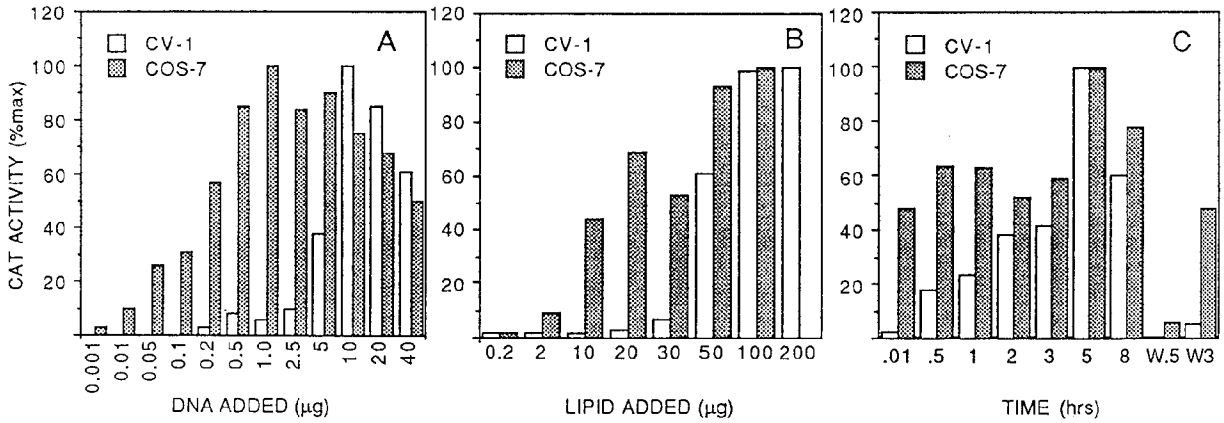

FIG. 3. CV-1 (stippled bars) and COS-7 (open bars) cells were transfected with pSV2cat complexed with PtdEtn/DOTMA (1:1). The standard transfection technique described in *Methods* was used, with the variations noted below. Cells were harvested after 2 days and CAT activity (per μg of cell protein) was determined. (*A*) Various concentrations of DNA with 100 μg of lipid were used in parallel transfections. (*B*) Various concentrations of lipid were used in parallel transfections with either 10 μg (CV-1) or 1 μg (COS-7) of DNA. (*C*) Cells were transfected using 100 μg of lipid and either 10 μg (CV-1) or 1 μg (COS-7) of DNA. After the time indicated, 10 ml of growth medium was added. W.5 and W3 represent parallel incubations where at the indicated time (0.5 and 3 hr, respectively), the lipid–DNA mixture was removed and replaced with 10 ml of fresh medium.

In CV-1 cells, maximal expression of CAT activity is achieved with 10 μg of DNA and there is a significant decrease with lower concentrations. In both CV-1 and COS-7 cells, very high DNA levels have some inhibitory effect. The difference in the dependence on DNA concentration between CV-1 and COS-7 cells probably lies with the ability of COS-7 cells to replicate pSV2cat to a high copy number. Presumably COS-7 cells are less dependent on the amount of DNA taken up by each cell than on the percentage of cells transfected. CV-1 cells, on the other hand, may be dependent on both the amount taken up by each cell and the transfection frequency.

Concentration of lipid. Increasing concentrations of lipid improve transfection of both CV-1 and COS-7 cells (Fig. 3*B*). However, the lipid is toxic to these cells at high levels (above 100 μg), and though an increase in specific activity of the cell extract can be obtained, the yield of enzyme activity decreases due to cell death. We have transfected a number of cell types that grow as monolayers, and in every case satisfactory transfection was obtained with subtoxic levels of lipid.

Time of incubation with DOTMA–DNA complexes. In standard transfections, cells were incubated with the lipid–DNA mixture for 3 hr. Growth medium was then added to the cells and, after a further 16 hr, this mixture was replaced with fresh medium. The length of the initial incubation period was varied, and the results are presented in Fig. 3*C*. The addition of growth medium after 5 min of treatment of CV-1 cells with the lipid–DNA mixture almost completely inhibits transfection. As the length of the treatment with the lipid–DNA complex increases, so does the transfection efficiency. An optimal transfection time of 5 hr was determined for both CV-1 and COS-7 cells. Incubations longer than 8 hr with lipid resulted in unacceptable levels of toxicity, but this may vary in other cell lines. Even after 3 hr of treatment, however, replacement with fresh medium yielded only 7% of the CAT activity obtained when medium was added to cells in the continuing presence of the lipid–DNA complex (Fig. 3*C*). We also observed that transfection of mouse L cells with DOTMA is inhibited by serum-containing growth medium. In particular, when serum was present from the outset, CAT activity was reduced to less than 5% of control (data not shown). COS-7 cells, in contrast to CV-1 cells and L cells, appear partially refractory to the presence of serum-containing growth medium (Fig. 3*C*). Furthermore, washing the cells after 3 hr of treatment with the lipid–DNA complex does not reduce the transfection efficiency, as measured by CAT activity, when compared to the addition of growth medium after 3 hr. We suspect that the relative insensitivity of COS-7 cells again reflects their ability to replicate pSV2cat to high copy number.

Lipofection vs. DEAE-Dextran and Calcium Phosphate for Transient and Stable Transfection of Cells. The ability of DOTMA to facilitate DNA uptake and functional expression was compared with the commonly used mediators of transfection, DEAE-dextran and calcium phosphate. In CV-1 and COS-7 cells, DOTMA yielded a 6- to 11-fold increase in CAT activity relative to transient transfection with DEAE-dextran (Table 1). A variety of cell lines, including the rat hepatoma HTC cell line, seem to be highly refractory to transient transfection with DEAE-dextran. In contrast, DOTMA complexes of pSV2cat yielded reproducible transfections of JZ.1 cells (a subclone of HTC cells), exhibiting at least an 80-fold enhancement over the DEAE-dextran procedure.

DOTMA is useful not only for transient transfection but can also be used to facilitate the stable transformation of cells. We analyzed the frequency of stable transfection of various cell lines with DOTMA complexes and compared it to the frequency obtained with calcium phosphate-precipitated DNA (Table 2). In four different cell lines, DOTMA yielded from 6 to greater than 80 times as many stable transformants, using either neomycin (G418) resistance or *E. coli* xanthine (guanine) phosphoribosyltransferase expression as the selectable markers.

Table 1. Transient transfections: Lipid compared with DEAE-dextran

Cell line	Lipid, μg	DEAE-dextran, mg/ml	Time, hr	DNA, μg	CAT specific activity, % maximal
JZ.1	100		3	5	19
	100		3	25	100
	150		3	25	80
		0.25	5	5	<1
		0.25	5	25	1
		0.25	16	25	<1
		0.50	5	25	1
CV-1	100		5	10	100
		0.25	5	10	9
COS-7	100		5	1	100
		0.25	5	1	7
		0.25	5	10	16
		0.50	5	1	7
		0.50	5	10	11

Transfections were carried out as described in *Methods*; lipid corresponds to PtdEtn/DOTMA (1:1). Each cell line represents an independent experiment, and in each case the transfection that yielded the highest level of CAT activity was set at 100%. All transfections were performed in duplicate, and CAT assays from each were performed in duplicate.

DISCUSSION

We have described the use of liposomes containing the cationic lipid DOTMA to facilitate the functional delivery of DNA into cells. The spontaneous formation of DOTMA–DNA complexes that are effective in DNA transfection suggests to us that a single plasmid is surrounded by sufficient cationic lipid to completely neutralize the negative charge of the DNA and provide a complex with a net positive charge that can associate with the negatively charged surface of the cell. The technique works well for both stable and transient expression of the introduced DNA, and with several cell types we have studied it is more efficient than either DEAE-dextran or calcium phosphate precipitation.

Table 2. Stable transfection: Lipid compared with calcium phosphate

Cell line	Plasmid	Method	Frequency × 10^5
L-M(TK$^-$)	pSV2neo	Calcium phosphate	3
		Lipid	45
ψ-2	pZIPSVX	Calcium phosphate	0.6
		Lipid	>49
ψ-2	pZIPC5a	Calcium phosphate	1.8
		Lipid	>68
MSN610.2	pSV2neo	Calcium phosphate	1.3
		Lipid	8.2
TA1	pSV2neo	Calcium phosphate	2
		Lipid	14
TA1	pZIPSVX	Calcium phosphate	0.7
		Lipid	17
TA1	pMSGC5a	Calcium phosphate	1.3
		Lipid	19

Cells were transfected with 7 μg of the indicated plasmid with no carrier DNA, except for pSV2neo where 1 μg of plasmid and 10 μg of carrier DNA were used. The transfection frequency is the number of drug-resistant colonies expressed as a fraction of the total number of cells plated.

The quantity of DNA required for an optimal signal in transient transfections varies with cell type. COS-7 cells, which replicate pSV2cat to a high copy number, require only 1 μg of DNA, whereas CV-1 cells require 10 μg. In both cases DNA levels above the optimum are inhibitory, although this effect is small. Indeed, with COS-7 cells, varying the DNA level from 0.2 to 40 μg gives a transfection signal that is never less than 50% of the maximum obtained. This, together with the observation that DOTMA-mediated transfection is effective for impure DNA preparations such as those obtained from crude bacterial lysates, would allow the rapid screening of new plasmid constructions by transfection. The smallest quantity of DNA required for a detectable signal depends to a large extent on the DNA and detection system used. With pSV2cat in COS-7 cells, 1 ng of DNA (without carrier) gives rise to an easily detectable CAT signal. Replacement of the SV40 early promoter with the Rous sarcoma virus promoter (pRSVcat) allows CAT enzyme levels to be detected with as little as 0.1 ng of DNA (J.P.N., unpublished observation). Moreover, with both pSV2cat and pRSVcat the addition of carrier DNA does not adversely affect the transfection signal obtained.

The concentration of lipid used in a transfection depends on the cell type. We have been able to obtain satisfactory transfection, both stable and transient, with DOTMA/PtdEtn levels between 50 and 100 μg. Increasing the lipid concentration above these levels may increase the specific activity of the cell extract, but significant toxicity also occurs. The toxicity varies with the type of cell, the duration of exposure to DOTMA, and the density of the cell culture; dense cultures are more resistant to the toxic effects of the lipid than less dense cultures. Although high levels of lipid are toxic, in our hands it appears to be less toxic than the concentration of DEAE-dextran that is required for optimal transfection of most cell types. Based on our experience it is best to optimize the various parameters described above for each cell line.

The exact composition of the DOTMA-containing liposomes can be varied, since pure DOTMA liposomes are almost as effective as DOTMA/PtdEtn (1:1) liposomes. If, however, the PtdEtn-containing liposomes are formulated with a negatively charged lipid, such as phosphatidylglycerol, rather than with DOTMA, transfection is completely abolished. Surprisingly, two commercially available cationic lipids which bear significant structural similarity to DOTMA, stearylamine and dioctadecyldimethylammonium bromide, have shown little efficacy as mediators of DNA transfection with mouse L cells (P.L.F., unpublished work). The properties of DOTMA-containing liposome that we have described here suggest that this method may be useful for introducing large DNA molecules, oligonucleotides, and RNAs into mammalian cells.

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